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Determination of Heparin-Induced Thrombocytopenia: A Rapid Flow Cytometric Assay for Direct Demonstration of Antibody-Mediated Platelet Activation

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Heparin-induced thrombocytopenia (HIT) and thrombosis are serious complications of heparin therapy. Recently, we have reported a practical and rapid functional flow cytometric assay (FCA) for the diagnosis of HIT with high specificity and sensitivity compared with the radioactive serotonin-release assay (SRA). In the present study, we added an immune-neutralization assay to directly demonstrate the antibody-mediated process, and tested the immune compatibility of low-molecular-weight heparin (LMWH) Lovenox and the heparinoid Orgaran (danaproid) using plasma from 18 patients with HIT confirmed by both FCA and SRA. The clinical utility of this modified method is demonstrated by a pediatric patient with a complex clinical presentation who developed thrombocytopenia with multiple thromboses while on heparin therapy. ELISA and SRA (performed in three independent laboratories) for diagnosis of HIT were both negative. In contrast, the FCA for detecting activated platelets expressing anionic phospholipids, was highly and reproducibly positive with both unfractionated and LMWH. Another FCA also demonstrated the surface expression of the α-granule membrane p-selectin (CD62p). Compatibility testing with the heparinoid Orgaran was also positive (and with plasma from 4 of the 18 patients with HIT). Heparin was discontinued, along with full recovery of the platelet count. The capacity of the patient's plasma to activate platelets in the presence of heparin gradually decreased over 4 weeks consistent with antibody clearance. The responsible mechanism was clarified using an immune-neutralization assay, which showed a dose response neutralization of the plasma activity by antibodies against human Immunoglobulin G (IgG) and IgM. This assay was also reproducible in the 18 patients with HIT. We conclude that the functional FCA with its modification is practical, sensitive, and specific for reliable diagnosis of HIT. It can simultaneously assess the compatibility of alternative therapies and directly confirm the antibody-mediated process. Further, it is particularly useful to clarify mechanisms of thrombocytopenia and thrombosis and to direct therapy in patients with a complex presentation and confounding laboratory results who often need prompt diagnosis and treatment. Am. J. Hematol. 61:53–61, 1999.

Key words: thrombocytopenia; thrombosis; heparin; flow cytometry; heparin-induced thrombocytopenia (HIT)

INTRODUCTION

Heparin-induced thrombocytopenia (HIT) and thrombosis are serious complications that may develop in patients sensitized to heparin. The immune-mediated mechanism involves the induction of antibodies against heparin-platelet factor-4 complexes, and the interaction of the heparin immune-complexes with platelets. This results in platelet activation and destruction [1–5]. Approximately 5% [1–5] to 30% [3,6] of patients treated with heparin develop HIT, which may be complicated by arterial and venous thrombosis caused by intravascular platelet activation and endothelial damage [7], resulting in serious morbidity and death. The diagnosis of HIT mandates the discontinuation of heparin. However, an

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effective alternative therapy is often needed immediately for patients who require anticoagulation [4]. Furthermore, alternative therapies such as low-molecular-weight heparin (LMWH) or heparinoids may cross-react with the heparin-induced antibodies, resulting in aggravation of the thrombocytopenia and thrombotic complications [4,5]. Thus, rapid and reliable documentation of HIT is required for clinical decision-making and patient management. Unfortunately, current assays used to determine HIT carry certain methodological or practical limitations. The platelet aggregation assay is considered to be relatively insensitive and nonspecific [1,2,7,8]. The serotonin-release assay (SRA) is sensitive and specific and is the recommended assay for HIT [1,2,4,6–8]. However, it uses radiolabeled reagents and requires considerable expertise to obtain reproducible and reliable results [1,2]. Moreover, the assay currently is not approved for clinical application outside of research laboratories [5], thus providing only retrospective confirmation. The enzyme-linked immunosorbent assay (ELISA) is sensitive and specific for detection of antibodies [9]. However, it is not approved currently for clinical use and is not cost-effective for individual assays. Moreover, the ELISA is a nonfunctional assay and thus cannot provide, by itself, unequivocal confirmation of the diagnosis of HIT [2,6,9]. Because of the lack of a rapid and reliable determination of HIT, initial diagnoses are presently made on a clinical basis, despite frequently complex patient presentations.

To overcome some of these difficulties, we recently developed a new functional flow cytometric assay (FCA) for the diagnosis of HIT [10]. The assay is practical and rapid, uses nonradioactive, readily available reagents, and uses standard equipment and methodology. It is highly sensitive and specific as compared with the SRA. The purpose of this work is to present the addition of an immune-neutralization assay to the original method to demonstrate directly that the patient's antibodies, in the presence of heparin, are implicated in the platelet activation process. The clinical utility of the FCA with the complementary assay is demonstrated by a patient with a complex clinical presentation associated with thrombocytopenia and thrombosis while on heparin therapy who was later discovered to have HIT by the FCA despite a negative SRA and ELISA. Finally, the feasibility of the method to test immune compatibility of alternative therapy such as LMWH and heparinoid is demonstrated also.

MATERIALS AND METHODS

Case Report

A 10-month-old female with congenital heart disease (pulmonary atresia with ventricular septal defect [VSD]) that required surgical intervention (Blalock-Taussig shunt) at the age of one week, was admitted for patch closure of the VSD and placement of a 16 mm homograft conduit from the right ventricle to pulmonary artery. The patient previously had received heparin for the initial surgery, angiographic studies, and for flushing of intravenous lines. Intraoperatively, she developed anaphylactic shock and cardiac arrest attributed to aprotinin, an antifibrinolytic agent used during cardiac bypass surgery. She was successfully resuscitated and placed on inotropic support and cardiac pacing for heart block. During the immediate postoperative period, the platelet count dropped from a baseline level of 425×10^9/l to a level of 117×10^9/l, along with an elevated International Normalized Ratio (INR) of 2.3, elevated fibrinogen degradation products >40 μg/ml (normal: <5) and low antithrombin III (ATIII) level of 58%. The activated partial thromboplastin time was 24 sec (normal: 19–33) and the fibrinogen was 263 mg/dl (normal: 180–420). The laboratory and intervention summary of the clinical course over three weeks is summarized in Table I. A presumptive diagnosis of disseminated intravascular coagulation (DIC) was considered and the patient was treated initially by replacement therapy including fresh frozen plasma and Vitamin K, and then by ATIII and platelet transfusion along with heparin infusion. The heparin was given initially for two days as a low-dose infusion (10 mg/kg/hr) to interrupt thrombin generation, and then as a therapeutic infusion (20–30 mg/kg/hr) for five days, to achieve therapeutic heparin levels of 0.3–0.6 U/ml. On day 6, she developed progressive cold, bluish extremities and there was inability to locate the radial, brachial, or dorsalis pedis arteries by palpation or doppler ultrasound. This clinical picture was compatible with arterial thromboses. An arteriogram of both upper extremities revealed bilateral small vessel occlusion. The ischemia worsened and the thrombocytopenia persisted reaching a nadir of 10×10^9 platelets/l. HIT was suspected because of the worsening clinical condition and persistence of thrombocytopenia despite supportive care, therapeutic heparinization, and ATIII replacement. Blood samples were drawn for the radioactive SRA, ELISA, and FCA for activated platelets. The SRA and ELISA were negative (performed at three independent laboratories), but the FCA was reproducibly positive. Accordingly, heparin was discontinued seven days after initiation and the platelet count gradually recovered to normal levels five days later (Table I). The patient, unfortunately, had to undergo amputation of both feet and the left hand because of extensive necrosis.

Laboratory Testing for HIT

FCA for diagnosis of HIT. Testing of the patient's plasma by the FCA developed in our laboratory was highly and reproducibly positive. The assay was performed according to the method recently reported [10]. Briefly, the patient's plasma was incubated with normal

TABLE I. Sequential Laboratory Values and Therapeutic Interventions in a Patient With Presumed HIT From Surgery (Day 1) Until Resolution of Thrombocytopenia*

Day	Platelets × 10^9/l	INR	PT sec	aPTT sec	Fibrinogen mg/dl	FDP μg/ml	D-Dimer ng/ml	Factor VII %	ATIII %	Heparin level (μ/ml)	Other laboratory testing	Intervention
1	425,000	1.2	13.9	24.2								Bypass surgery, heparin
2	117,000	2.3	19	24	263	>40			58			
3	112,000	4.7	27	37								Vitamin K
4	61,000	6.1	31	39								FFP
5	21,000	5.9	30	38								FFP, Plt, heparin
6	11,000	2.7	21	26			8-16,000		55		Protein C = 35%	Arteriogram, heparin, ATIII, Plt
7	10,000	2.3	19	30	247	>40	4-8,000	17	125	0.16	Factor VIII = 311%	Heparin, ATIII, Plt
8	21,000	2.3	19	41	372	<10	1-2,000	17	115	0.6	Protein C = 40%; Protein S = 56%	Heparin, ATIII, Plt
9	41,000	2.0	18	54	406	<10	1-2,000	19	145	0.18		Dextran; Plt D/C, ATIII, heparin
10	43,000	1.9	17	62	402	<10	1-2,000	24	76	0.24		Heparin, ATIII
11	49,000	1.6	16	73	333	10–40	0.5-1,000	34	162	0.4		Heparin and ATIII D/C
12	61,000	1.4	15	25	339	<10	0.5-1,000	50	83	0.05	Protein C = 51%	
13	53,000	1.3	14.6	24	348	<10	250-500	50	93		Protein C = 67%	
14	72,000	1.5	15.6	25	437	<10	0.5-1,000					
15	83,000											
16	136,000											
17	130,000											
18	161,000											
19	182,000											

*HIT, heparin-induced thrombocytopenia; INR, international normalized ratio; PT, prothrombin time; aPTT, activated partial thromboplastin time; FDP, fibrin degradation products; ATIII, antithrombin III; FFP, fresh frozen plasma; Plt, platelet transfusion; D/C, discontinued. Normal ranges: Platelet count = 150–400 × 10^9/l; PT = 10.6–14.1 sec; aPTT = 22–34 sec; fibrinogen = 200–400 mg/dl; FDP < 5 μg/ml; D-dimer < 250 ng/ml; factor VII = 50–150%; ATIII = 80–120%; protein C = 38–80%; protein S = 66–120%.

platelet-rich plasma (PRP) in the presence of two pharmacological concentrations (0.1 and 0.3 IU/ml) of standard unfractionated porcine heparin (UH) (Elkins, Sinn Company, Cherry Hill, NJ), LMWH, Lovenox, and the heparinoid Orgaran (see below). Total reaction volume was 100 μl. Internal controls with anticipated negative results contained: a. no heparin, to exclude a direct effect of plasma on platelets and to confirm the necessity of heparin for the reaction, and b. 100 IU/ml heparin to confirm the immune-mediated mechanism of HIT, which can be abolished by excess of antigen, and to exclude a direct effect of free heparin on platelet activation. Positive control samples obtained from patients with HIT confirmed by clinical history, SRA, ELISA, and FCA were also included. In vitro stimulated platelets with calcium ionophore A23817 were used for quality control. Plasma samples were also tested simultaneously with the FDA-approved LMWH Lovenox (Rhone-Poulenc Rorer Inc., Collegeville, PA), and the heparinoid Orgaran (danaproid, Organon Inc., West Orange, NJ) to assess immune cross-reactivity with the patient's plasma. Following the initial incubation step, 5 μl aliquots from each sample were further incubated with phycoerythrin (PE)-labeled monoclonal antibody (MoAb) against platelet glycoprotein IIb/IIIa (CD41a) (P2 clone; Immunotech Inc. [AMAC], Westbrook, ME) for platelet identification, and with directly fluoresceinated recombinant annexin V (Dr. Toru Yokoyama, Kowa Co., Tokyo, Japan) for detection of activated platelets (total volume 50 μl). The annexin V protein interacts with the prothrombinase-binding anionic phospholipid sites exposed on the platelet surface upon activation (platelet procoagulant activity). The platelet suspension was then diluted 1:10 by HEPES buffer and analyzed by flow cytometry.

The flow cytometric analysis was based on our previous experience for detecting in vivo and in vitro platelet activation [10–17] with an attempt to simplify and standardize the method. Platelet suspensions were analyzed by standard two-color flow cytometry using FACScan flow cytometer (Becton Dickinson, San Jose, CA) (e.g., Fig. 1). The platelet population was initially identified by light scatter, and was further distinguished from electronic noise and cell debris by the specific red-immunofluorescence of PE-labeled anti-GPIIb/IIIa MoAb. The platelet population was then electronically selected and analyzed for activation by the high intensity of the annexin V green-fluorescence. The fraction of activated platelets was immediately determined and com-

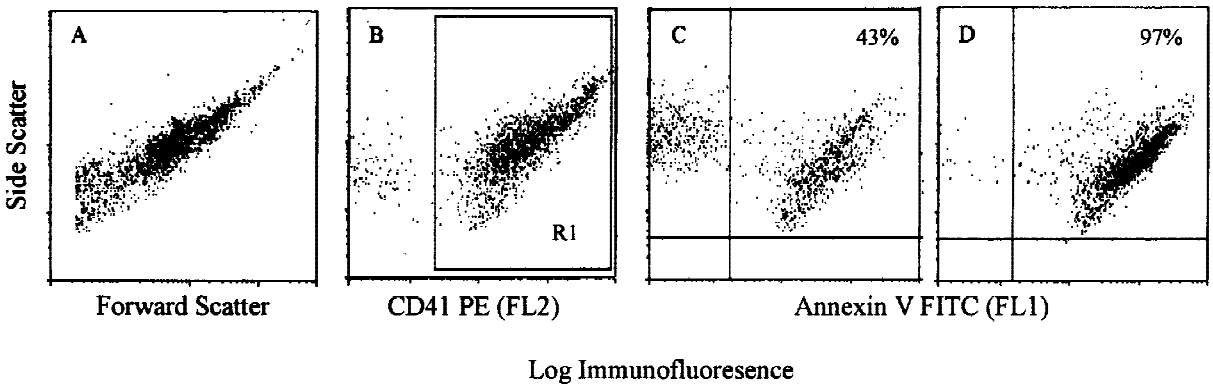

Fig. 1. Flow cytometric detection of platelet activation. Platelets were initially detected by the characteristic light scatter (A), and further resolved from electronic noise and cell debris by the red-immunofluorescence (FL2) of PE-labeled anti-GPIIb/IIIa MoAb (B, box R1). Activated platelets were identified by the green fluorescence (FL1) of FITC-labeled annexin V, and their fraction was directly determined by setting a marker (C, vertical line) between the activated (43%) and the nonactivated subpopulations. The right panel (D) shows maximal activation (97%) induced in vitro by calcium ionophore.

pared to 1. the negative internal controls (incubated with 0 and 100 IU/ml heparin) to confirm specificity, and 2. the normal control to assess positivity. Ten thousand platelets were analyzed per each sample using the LYSIS program (Becton Dickinson).

Testing for α-granule release reaction. For further confirmation of the results obtained with the regular FCA (testing for platelet activation by the expression of anionic phospholipids), platelets also were tested by flow cytometry for α-granule release (Fig. 3). Following the incubation of PRP with patient plasma and heparin, platelets were further incubated with fluorescein (FITC)-labeled MoAb GA6 (Biogen, Cambridge, MA) which reacts with the α-granule membrane glycoprotein p-selectin (CD62p) translocated to the platelet surface upon activation.

Antibody-neutralization assay. To demonstrate directly that platelet activation induced by patient plasma is an antibody-mediated process, a complementary test was added to the functional FCA. The patient's plasma was incubated, prior to the FCA, with neutralizing antibodies directed against human Ig. Incubation was performed with goat-antihuman (GAH) Ig, GAH IgG, IgM, and IgA preparations, and goat-antimouse (GAM) antibodies as a negative control (Immunotech). Briefly, 20 μl of plasma was incubated with GAH antibodies (Fig. 5) in 50 μl final volume for 30 min at room temperature, then tested for the capacity of the plasma to activate platelets in the presence of heparin, using the regular functional FCA.

ELISA assay. An ELISA for determination of antibodies in the patient's plasma against heparin-platelet factor 4 complex was performed according to the manufacturer's instructions (ESSECHROM HIPA, American Bioproducts Co., Parsippany, NJ).

Serotonin-release assay (SRA). The radioactive assay was performed in three independent laboratories (including the author's) according to Sheridan et al. [1] and Kelton et al. [2].

Statistical Analysis

Regression analysis was used to define the cut-off value for maximal separation between negative and positive results, and the related sensitivity and specificity [10]. A positive result was defined as a value (% activated platelets) greater than the mean of normal control +2 standard deviations.

RESULTS

Platelet Count

The circulating platelet count in relationship to treatment is shown in Table I. On the first day postcardiac by-pass surgery, the platelet count dropped from 425 × 10^9/l to a level of 117 × 10^9/l, eventually reaching a nadir of 10 × 10^9/l while on heparin therapy. Following discontinuation of heparin, the platelet count recovered rapidly, reaching a normal level within 5 days.

Testing for HIT

The results of the ELISA assay, routinely performed at the clinical laboratory, were reported as negative. The results of the radioactive SRA, performed in three independent laboratories, were also negative. In contrast, the functional FCA was highly and reproducibly positive. Figure 2 demonstrates the activation of platelets by patient plasma in the presence of UH (panel B). Incubation of normal plasma (NC, panel A) with 0, 0.3, and 100 IU/ml heparin or of patient plasma with 0 and 100 IU/ml heparin, produced a background level of about 4% activated platelets. Only the patient's plasma (Pt, panel B) incubated with pharmacological concentration (0.3 IU/

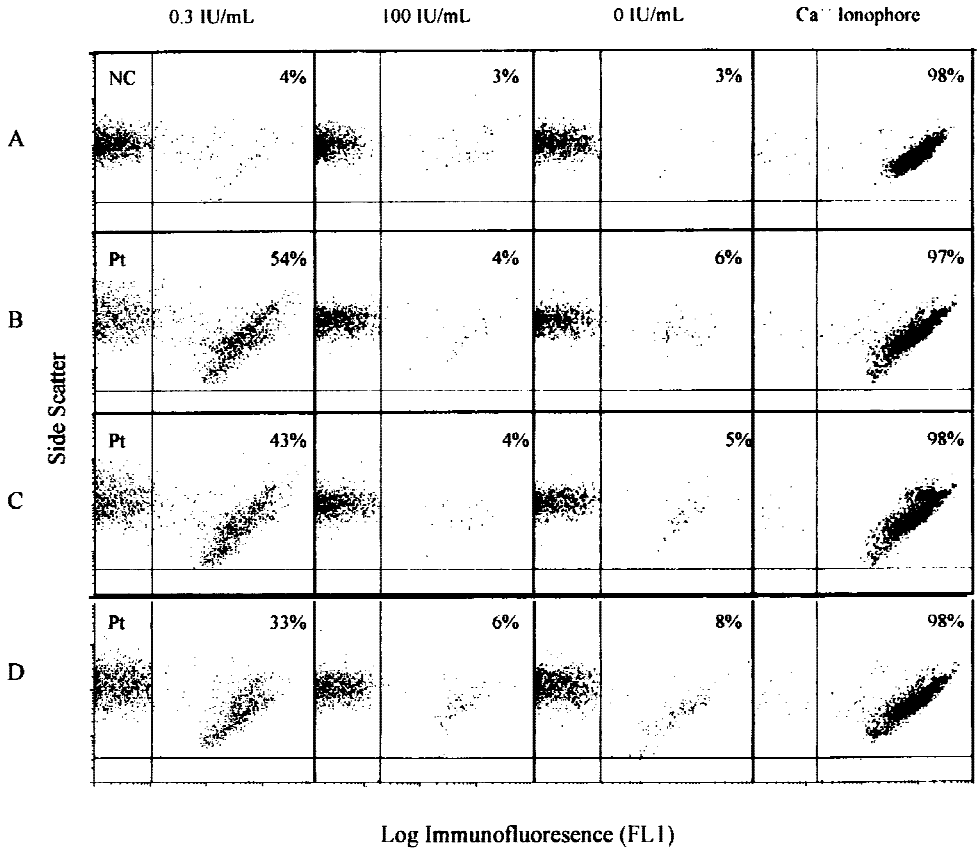

Fig. 2. Flow cytometric determination of HIT. Patient's plasma (Pt) was incubated with PRP in the presence of pharmacological concentration of unfractionated heparin (0.3 IU/ml) (panel B), an excessive dose of heparin (100 IU/ml), and without heparin. Platelet activation was detected only with 0.3 IU/ml heparin (54% of platelets analyzed), confirming the specificity of the reaction at pharmacological concentrations. Incubation of PRP with normal control (NC) plasma showed no activation (panel A). Compatibility testing with LMWH Lovenox (panel C) and heparinoid Orgaran (panel D) showed positive results. The right panels show maximal activation induced by calcium ionophore.

ml) of heparin resulted in significant activation of 54% of platelets analyzed. Incubation with 0.1 U/ml (not shown in the Figure) resulted in 48% activated platelets. By comparison, the addition of calcium ionophore resulted in maximal platelet activation of 97–98% (right panels). These results demonstrate the specificity of the reaction at pharmacological concentrations of heparin. The fact that the reaction can be abolished by a large excess (100 IU/ml) of the antigenic heparin molecules (Fig. 2, panels B, C, and D) provides direct support for the immunologic nature of the reaction, which requires aggregation of the patient's antibodies by the heparin macromolecules.

The results obtained with LMWH were concordant with those found with UH (Fig. 2, panel C), demonstrating the interaction of the patient's antibodies with LMWH with subsequent platelet activation. The patient's antibodies induced by unfractionated heparin, therefore, cross-react with LMWH. Testing of patient plasma with the heparinoid Orgaran (danaparoid), which is considered a preferable alternative therapy for patients with HIT who require anticoagulation, was also positive, as demonstrated in Figure 2 (panel D). These results suggest that both LMWH and Orgaran might not present alternative treatment options for this patient.

The assay with LMWH was also reproducible in another 18 patients who previously tested positive for HIT by both FCA and SRA. These patients were clinically suspected for HIT and their platelet counts recovered rapidly after the discontinuation of heparin. As shown in Figure 2, incubation with LMWH resulted in somewhat lower degree of platelet activation compared with unfractionated heparin (mean value = 22.2 vs. 27.8). The immune-compatibility testing for Orgaran was positive in four of these patients.

The confirmatory flow cytometric evaluation for α-granule release induced by patient plasma in the presence of heparin is shown in Figure 3. After incubation, platelets were activated, expressing the α-granule membrane glycoprotein p-selectin (CD62p) on the surface membrane.

To clarify further the nature of the patient's plasma activity in relation to therapy, four sequential samples

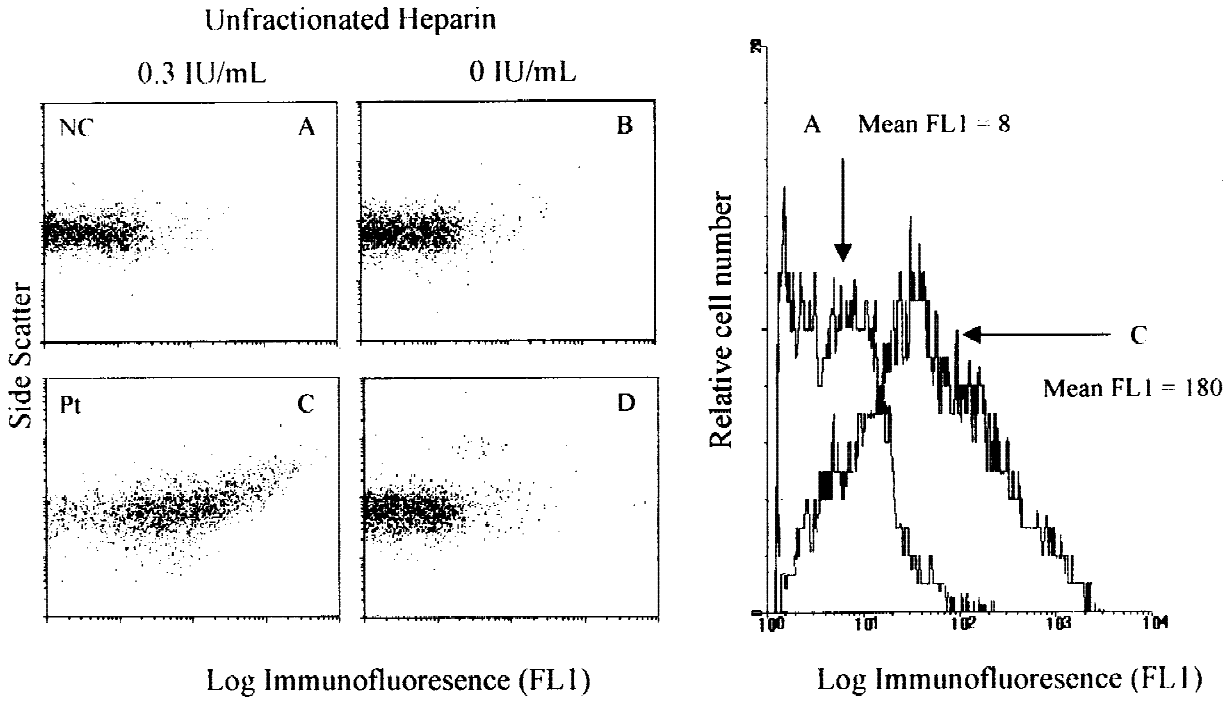

Fig. 3. Flow cytometric detection of α-granule release. Release reaction induced by patient's plasma in the presence of heparin was detected by incubation with FITC-MoAb GA6 (FL1). This antibody reacts with the α-granule membrane glycoprotein p-selectin (CD62p) translocated to the surface membrane upon release. The mean fluorescence values of the samples shown in panels A (NC) and C (patient's sample, Pt) of the dot plot histograms, are presented in the single-parameter histograms A and C shown on the left.

were obtained in a 4-week interval after discontinuation of heparin and normalization of platelet counts and coagulation tests. As shown in Figure 4, the plasma capacity to induce platelet activation in the presence of heparin decreased to about 50% in 2 weeks and to 15% of the initial activity in 4 weeks. These results are consistent with clearance of circulating antibodies.

Antibody-Neutralization Assay

To demonstrate directly that the platelet activation induced by patient plasma in the presence of heparin is an antibody-mediated process, complementary testing was added to the functional FCA. As shown in Figure 5, the preincubation of patient plasma with antihuman Ig antibodies resulted in suppression of platelet activation in a dose-response manner. The suppression of the plasma activity was observed with GAH-Ig, GAH-IgG, and GAH-IgM, but not with GAH-IgA or GAM Ig preparations. Thus, these results demonstrate that the capacity of the patient's plasma to induce platelet activation in the presence of heparin is mediated by the patient's antibodies composed of IgG and IgM classes. The antibody-neutralization assay was also reproducible with all other 18 plasma samples previously found positive for HIT by both the FCA and the SRA (mean percent neutralization = 76.2 ± 4.3).

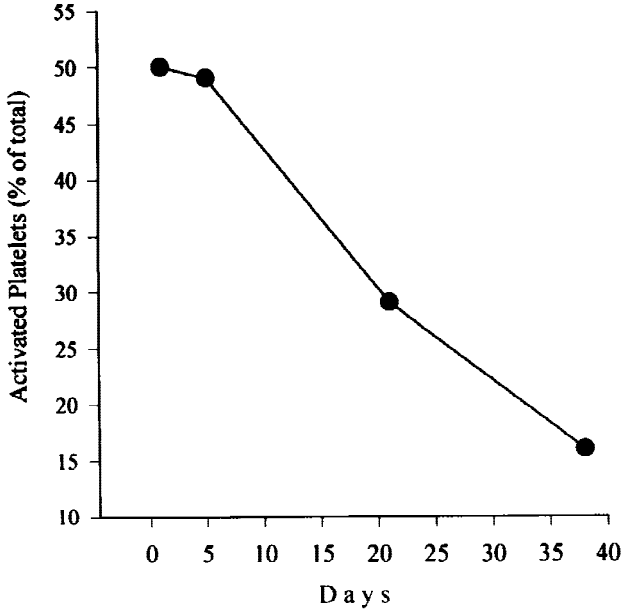

Fig. 4. Time course of the patient's plasma capacity to induce platelet activation. The ability of the patient's plasma to activate platelets in the presence of heparin gradually decreased over 4 weeks consistent with antibody clearance. The flow cytometric tests were performed as shown in Figures 1 and 2, and the percent of activated platelets in each sample tested was plotted against time.

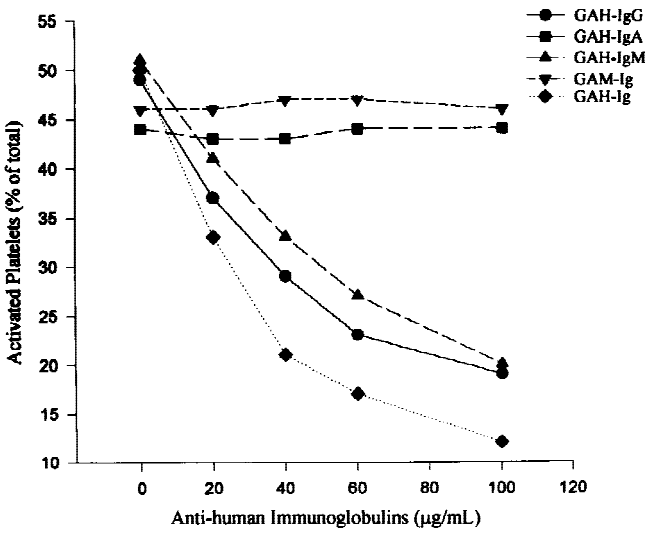

Fig. 5. Neutralization of the patient's plasma capacity to induce platelet activation in the presence of heparin is shown. Preincubation of the patient's plasma with antihuman Ig suppressed the heparin-mediated platelet activation in a dose-response manner. Suppression was demonstrated with GAH Ig, GAH IgG, and IgM preparations but not with GAH IgA or GAM Ig.

DISCUSSION

In the present study, we demonstrated the usefulness of the functional flow cytometric method for determination of HIT to clarify the mechanism of thrombocytopenia and thrombosis in a patient with a complex clinical presentation and negative SRA and ELISA assays. The method was found to be sensitive and specific (100%, respectively) and highly correlated with the established SRA (95% sensitivity and 100% specificity as compared with the SRA) [10]. The method is practical and rapid, able to support effectively clinical decision making and patient management. To enhance the FCA diagnostic capacity, we added a complementary immune-neutralization assay that can be performed simultaneously with the standard FCA. The results obtained in the study patient and in 18 patients who previously tested positive for HIT, demonstrated that the platelet activation process is mediated by circulating antibodies. Additionally, the feasibility of the method for simultaneous immune compatibility testing of alternative therapy such as LMWH and heparinoid was also demonstrated.

In the study patient, the initial clinical and laboratory presentation was complicated by findings suggestive of DIC, leading to treatment incompatible with that for HIT. Initially, heparin at a lower dose of 10 U/kg/hr, was added in an attempt to interrupt the presumed tissue factor release and excessive thrombin generation. However, despite this treatment, the patient developed arterial thromboses in all extremities with a further decrease in the platelet count on the sixth postoperative day. Escalating heparin to a full therapeutic dose was followed by clinical worsening of the ischemia, thus suggesting the diagnosis of HIT. Cessation of heparin was delayed because of negative testings for HIT by the ELISA and SRA (performed in three independent laboratories). However, because of continued worsening of the ischemia and the positive FCA (both supporting a diagnosis of HIT), heparin was discontinued along with full recovery of the platelet count within 5 days.

In retrospect, the platelet count did not recover above the mid-40,000 $\times 10^9$/l range, despite near normalization of the FDP and D-dimer levels, until heparin was stopped. The nadir, which occurred several days earlier, coincided with the development of thrombosis. For these reasons and because the clinically apparent thromboses had not developed in association with the initial manifestations of DIC (for the initial 6 days), we believe that the overall data are consistent with arterial thromboses, which developed as a result of HIT. It is noteworthy that such a confounding clinical presentation is not unusual. Warkentin and Kelton [19] report that approximately 5% of patients with HIT may have concomitant hypofibrinogenemia, which is often associated with DIC. As discussed previously, the development of arterial thrombosis 6 days postoperatively while on heparin therapy and the recovery of the platelet count after the discontinuation of heparin are certainly consistent with the diagnosis of HIT. Moreover, the flow cytometric testing of four sequential plasma samples obtained over a 4-week interval demonstrated a gradual decrease in heparin-induced platelet activation, consistent with the mechanism of antibody clearance. Finally, the immune-neutralization study confirmed that the capacity of the patient's plasma to activate platelets in the presence of heparin is mediated by the patient's antibodies. This assay obviates the need for purification of the patient's antibodies.

The discrepancy found in this study between the positive results of the FCA (consistent with the deteriorating clinical condition of thrombosis and ischemia) and the negative results of known established tests for HIT such as the SRA and ELISA, is intriguing. Similar to the SRA (and in contrast to the ELISA), the FCA is a functional assay resulting in platelet activation and destruction. A previous study of 25 patients [10] showed three patients who were positive by FCA but negative by SRA. This finding may reflect a higher sensitivity of the FCA but may also indicate a difference in the mechanisms tested. For those three patients, as well as for the patient in this report, testing was positive for the induction of the platelet release as assessed by expression of the α-granule glycoprotein p-selectin (CD62p). Thus, a plausible explanation for the discrepancy between a positive FCA but a negative SRA is that the dense-granule (containing serotonin, ADP, and ATP) release reaction requires a stronger stimulus than that required for the release of α-gran-

ule constituents [21]. However, the precise mechanism responsible for these results is not clear. Differences in the sensitivity of detecting various reaction steps involved in the platelet activation process may explain the discrepant results.

The mechanism responsible for the discrepancy between the FCA and the ELISA is less clear. It is conceivable that the negative ELISA results are related to the false-negative rate of the assay. A very recent study of 105 serum samples from patients with HIT showed that the sensitivity and specificity of the ELISA, similar to the one used in the present study, was 71% and 76%, respectively when compared with the SRA [28]. In another comparison of the commercial ELISA with SRA [9], 5% to 10% of patients who were discovered positive by the SRA, had negative ELISA testing. No clear explanation was provided for this discrepancy. However, in another study, 15 of 87 patients with HIT, lacked antibodies to heparin-platelet-factor 4 (PF4) tested by an ELISA [29]. Patients from this study showed antibodies to PF4-related chemokines, neutrophil-activated peptide-2, interleukin-8, and platelet basic protein. These data are further highlighted by the observation that some patients with a positive SRA may not have antibodies detectable by the usual immunologic assays [2,6,9]. Thus, in general, functional assays are considered clinically superior to the immune-detection assays, because the latter detect only the presence of antibodies without establishing a causal relationship to platelet activation and destruction. This is illustrated by one study comparing the ELISA vs. the SRA assay [9] demonstrating that 22% of samples found positive by the ELISA were negative by the SRA. Thus, it has been suggested that a positive ELISA result alone cannot provide unequivocal confirmation of the diagnosis of HIT, and that the SRA should be performed to provide confirmatory evidence in such cases [9]. Based on the present data, we believe that the functional FCA, which like the SRA uses intact, functional platelets, can also provide the necessary confirmation for HIT.

We also addressed the compatibility of alternate anticoagulant therapy for patients with HIT, using the FCA. The results obtained with the LMWH Lovenox, were concordant with those obtained with unfractionated heparin, suggesting that LMWH may not be a practical alternative therapy for patients with HIT [5]. Moreover, as shown in Figure 3, testing of patient plasma with the heparinoid Orgaran (danaparoid), which is often considered a preferable alternative therapy for patients with HIT who require anticoagulation [4,5], was also positive (as well as in four of the other 18 previously described patients with HIT). These conclusions are consistent with previous observations and recent reports of thrombosis associated with severe morbidity and mortality in patients with HIT who received LMWH without compatibility testing [5,9,22–25]. In addition, it has been reported that in up to 10% of patients with HIT induced by unfractionated heparin, an immune cross-reaction with Orgaran may also occur, with possible serious consequences, including death [26]. Therefore, it is advisable that Orgaran not be used in patients with HIT without prior in vitro testing [4,22,23,26,27].

CONCLUSIONS

Our findings suggest that the functional FCA with its present modifications is a practical, sensitive, and specific test for reliably confirming the diagnosis of HIT, and for assessing the compatibility of alternative therapies. This information can be provided rapidly to support the management of patients with HIT who often need prompt diagnosis and treatment. In addition, the immune-neutralization assay performed simultaneously, may be useful to clarify further the mechanism of thrombocytopenia and thrombosis in patients with a complex clinical presentation and confounding laboratory results.

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